Size and domain structure of collagen VI produced by cultured fibroblasts

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Disulfide-bonded forms of collagen VI were analyzed by immunoblotting of fibroblast culture medium and cell extracts. The protein consists of pepsin and collagenase-resistant domains of about equal size indicating a molecular mass of 340 kDa for collagen VI monomers.

Microfibrillar collagen VI *Immunoblotting* *Fibroblast culture medium* *Domain structure*

1. INTRODUCTION

Collagen VI has been recently identified as a particular type of collagenous protein which forms microfibrils and cross-banded fibers with 100 nm periodicity in tissues and cell cultures [1–4]. It shows ubiquitous tissue distribution [2,5,6] and was originally discovered as a pepsin-resistant fragment obtained from aortic intima [7]. These pepsin fragments consist of a 105 nm long triple helical domain with globular domains at each end which are folded from 3 chain fragments of M_r = 40 000–70 000 [1,8–10]. The dumbbell-like monomers are usually assembled into dimers, tetramers and larger associates which are cross-linked by disulfide bonds [1]. The application of non-degrading extraction conditions led more recently to the chemical and immunological characterization of 110–140-kDa chains which are presumably the constituents of genuine tissue forms of collagen VI [2,6,11–16]. These genuine structures have the same dumbbell-like shape as found for pepsin-treated monomers and oligomers except for a distinctly larger size of both terminal globular domains [2,13].

Collagen VI is synthesized by fibroblasts and some other connective tissue cells. The chains of newly synthesized collagen VI are identical to or slightly larger in size than those found in tissues [2,6,12,14]. Controversial data exist about the nature of the globular domains, which have been reported to be of different size classes [14] or to be completely degraded by bacterial collagenase [15]. Since both sets of data are not compatible with structural data obtained for tissue forms of collagen [2,13] they prompted us to reexamine the domain structure of fibroblast-derived collagen VI.

2. MATERIALS AND METHODS

Fibroblast cultures from human embryonic or adult skin (4th to 10th passage) were grown to confluency in Dulbecco's modified Eagle's medium supplemented with sodium ascorbate (50 μg/ml), glutamine (300 μg/ml), penicillin (400 units/ml), streptomycin (50 μg/ml) and 10% fetal calf serum [17]. Medium was then collected from a 24 h incubation in the absence of serum. Cell layers were extracted with phosphate-buffered saline pH 7.2 by freeze-thawing and the insoluble pellet was extracted (18 h, 20°C) with 0.01 M cysteine, pH 7.2. Protease inhibitors (0.5 mM phenylmethane-sulfonyl fluoride, 0.1 mM *p*-hydroxymercuribenzoate) were added to the medium and the cell extract.

Proteins of the medium and cell extract were concentrated by precipitation with 30% saturated ammonium sulfate (fractions M-30 and C-30, res-

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pectively), precipitates were collected by centrifugation (12 000 rpm, 1 h), dissolved in a small volume of 0.5 M NaCl, 0.05 M Tris-HCl, pH 7.4, and after dialysis against the same buffer stored at −20°C. A second fraction (M-80) was obtained from the medium by raising the ammonium sulfate concentration to 80% saturation. Aliquots of these samples were dialyzed against 0.5% acetic acid adjusted to pH 2, and digested with pepsin (0.1 mg/ml) for 18 h at 4°C. The reaction was stopped by adding pepstatin (0.2 mg/ml) and neutralization with Tris base. After addition of 50 mM $CaCl_2$ and 2.5 mM *N*-ethylmaleimide other aliquots were digested with 1 μg/ml purified bacterial collagenase (kindly given by Dr B. Peterkofsky [18]) for 3–24 h at 37°C. The reaction was stopped by adding electrophoresis sample buffer and boiling (3 min). Collagenase digests were prepared from non-reduced samples and samples which were reduced with either 0.02 M 2-mercaptoethanol or 0.02 M dithiothreitol (4 h, room temperature) followed by alkylation with 0.08 M iodoacetamide for 2 h. Reagents were removed by dialysis. Control experiments without added enzymes revealed no change of the size of collagen VI chains as shown by immunoblotting.

Proteins were separated by polyacrylamide gel electrophoresis in the presence of SDS [19] prior to and after reduction with 5% 2-mercaptoethanol. They were then transferred to nitrocellulose [20] and collagen VI antigens were visualized by immunoblotting [21]. In some cases transfers were done after incubating the gels in 0.01 M dithiothreitol, pH 8.6 (1 h, room temperature). Affinity-purified rabbit antibodies against pepsin-treated collagen VI from human placenta [2] were used at a concentration of 15–35 μg/ml in the immunoblot reaction. Runs were calibrated with a collagen I standard and pepsin-treated collagen VI [10] and as non-collagenous markers with myosin, β-galactosidase, phosphorylase *b*, serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme obtained from Bio-Rad.

Medium proteins were separated by sucrose gradient centrifugation [22] in the presence of ^{125}I-labelled laminin [23] as internal marker. The fractions were analyzed for procollagen III by radioimmunoassay [24] and for collagen VI by immunodotting and after reduction and electrophoresis by immunoblotting.

3. RESULTS

Collagen VI antigen could be detected by immunoblotting in fibroblast culture medium only after concentrating it 50–100-fold by ammonium sulfate precipitation. Medium fraction M-30 (0–30% ammonium sulfate) showed a single band after reduction (140 kDa according to globular standards) which as shown previously [2] co-migrated with non-degraded collagen VI chains in tissue extracts. A double band of similar mobility was observed in the cell extracts obtained with or without cyteine (C-30). These samples showed no reaction or only a weak positive one when analyzed prior to reduction (fig.1 and 2). The medium fraction M-80 (30–80% ammonium sulfate) showed already prior to reduction a major 140-kDa band and in addition a component with the migration of a dimer (β-component). The latter was converted to the monomers by reduction (fig.1).

Disulfide bonding of M-30 components was studied by electrophoresis under non-reducing conditions followed by exposure to dithiothreitol prior to the transfer to nitrocellulose. Antibody staining then revealed a strong band on top of the

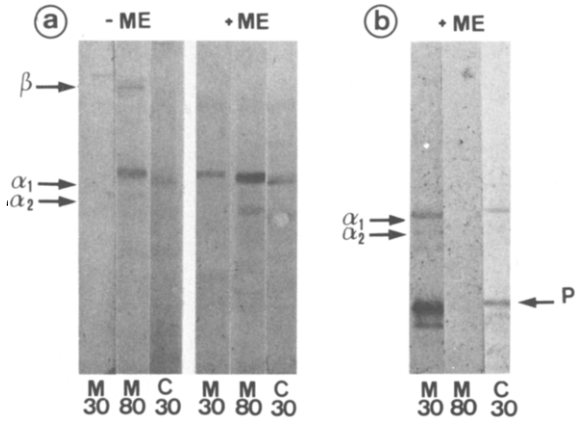

Fig.1. Identification by immunoblotting of non-degraded (a) and pepsin-treated (b) forms of collagen VI produced by fibroblasts. The collagens were 50–100-fold concentrated by precipitation with ammonium sulfate from culture medium (M-30, 0–30% saturation; M-80, 30–80% saturation) and from NaCl extracts of cells (C-30) and separated prior to (−ME) or after (+ME) reduction with 2-mercaptoethanol on 5–10% polyacrylamide gradient gels. Positions of collagen I markers (α1) and α2 chains, β-components) and of pepsin-treated collagen VI (P) are indicated by arrows.

gel indicating that the large size of the antigen had presumably prevented its efficient transfer to nitrocellulose prior to reduction. Treatment of fraction M-80 in the same way failed to demonstrate collagen VI antigens of high M_r. Separation of M-30 proteins by sucrose gradient centrifugation demonstrated cosedimentation of collagen VI with the added laminin marker which both appeared larger than procollagen III determined in the fractions by radioimmunoassay. Reduction of the collagen VI peak fraction produced the 140-kDa band on gel electrophoresis as found for the whole medium.

Treatment of fractions M-30 and C-30 with pepsin degraded the collagen VI antigen to one or two components which have the same mobility as the major bands of pepsin-treated collagen VI obtained from tissue samples (fig.1b). This indicates an M_r of about 40 000 for the fragments [8–10] which contain mainly the triple helical domain of collagen VI. The collagen VI component in fraction M-80 was entirely degraded by pepsin since no antigen bands could be detected by immunoblotting (fig.1b).

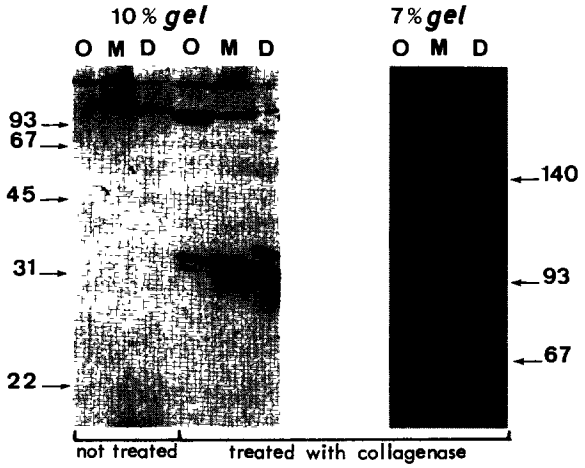

Fig.2. Immunoblot reaction of collagen VI domains resistant against bacterial collagenase. Materials were not reduced (O) or reduced with 0.02 M 2-mercaptoethanol (M) or with 0.02 M dithiothreitol (D) prior to treatment with collagenase for 3 h (10% gel) or 24 h (7% gel) and were compared to non-treated controls. The 7% gel run was aimed to show intermediary cleavage products and major fragments (below 50 kDa) have already run out from the gel. All separations were calibrated with globular marker proteins and their migration positions are labelled by their molecular masses given in kDa.

As expected from previous studies [19] digestion of collagen VI in fraction M-30 with bacterial collagenase was dependent on the partial or complete opening of disulfide bridges in the protein. Treatment with 0.02 M 2-mercaptoethanol or more efficiently with 0.02 M dithiothreitol allowed the degradation to a major doublet band with an average $M_r = 37\,000$ according to globular standards (fig.2). In addition mercaptoethanol-treated but not dithiothreitol-treated samples still contained a major 100-kDa band and some non-degraded 140-kDa material. The latter sample also showed a minor 42-kDa band which, however, was lost after a second reduction. Similar fragments but with much lower intensity were observed in collagenase-digests of non-reduced collagen VI.

4. DISCUSSION

We have recently characterized antibodies against pepsin-solubilized collagen VI from human placenta which react with collagenous and non-collagenous epitopes of all constituent chains of the antigen [2]. These antibodies were therefore suited for the domain analysis of intact collagen VI and demonstrated a single or two peptide chains in culture medium and cell layer extracts of skin fibroblasts. These peptide chains comprise in mobility collagen VI material characterized in other cell cultures [6,12,15] but are smaller than those found in bovine fibroblasts [14]. The peptide chains of fibroblast collagen VI are connected by disulfide bonds to larger complexes as shown by electrophoresis and sedimentation analysis. Most of the material sediments together with laminin ($s^0_{20,w} = 11.5\ S$; M_r about 1 000 000) and before procollagen III ($s^0_{20,w} = 4.2\ S$; $M_r = 450\,000$) indicating that it consists of collagen VI tetramers or larger aggregates [1]. Filamentous aggregates resembling collagen VI structures have been recently demonstrated in fibroblast cultures [3].

In addition, fibroblast medium contained smaller collagen VI components (fraction M-80) similar to those which have been isolated with monoclonal antibodies [6]. This material apparently consisted of non-triple helical monomeric and dimeric peptide chains which could be completely destroyed by pepsin treatment.

Collagen VI from fibroblasts possesses distinct collagenase-resistant peptide segments ($M_r =$

37 000) which are larger than those found in pepsin-treated collagen VI [10] and require reduction of disulfide bonds for their proper release. They very likely are the constituents of the two globular domains seen at each end of genuine collagen VI by electron microscopy [2,13]. These non-collagenous peptides showed a limited electrophoretic heterogeneity which could be due to their origin from different chains or different ends of the molecule. A collagenase-resistant peptide with $M_r = 38\,000$ but also two larger components ($M_r = 65\,000$ and $M_r = 76\,000$) have been described for reduced and alkylated collagen VI chains from bovine aorta [13]. Even larger fragments ($M_r = 100\,000$) were found after treating non-reduced collagen VI with collagenase [14] and, as shown by our data, are presumably incompletely cleaved fragments. Complete cleavage is prevented by the formation of collagen VI oligomers which stabilizes the overlapping triple helical segments [1,10]. Still another study [15] failed to identify large fragments in colagenase digests of reduced collagen VI. Since these data are incompatible with the presence of distinct globular domains in collagen VI they could reflect the lack of some antisera to react with these structures in immunoblotting.

Monomeric collagen VI has been visualized as a symmetric, dumbbell-shaped structure by rotary shadowing electron microscopy [2,13]. The data for the collagenase-resistant segments suggest an M_r of about 110 000 for each globular domain which together with $M_r = 120\,000$ for the triple helical domain [8–10] adds up to $M_r = 340\,000$ for intact collagen VI. This value is within the range predicted from $M_r = 110\,000$–$140\,000$ for the constituent chains [2,6,11–13,15,16] and in agreement with electron microscopic data indicating an M_r of about 150 000 for the globular domains [4]. The data do not support a structural model for collagen VI consisting of two globular domains of distinctly different size [14].

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